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Computational Design of TrkB Peptide Inhibitors and Their Biological Effects on Ovarian Cancer Cell Lines

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Abstract There are large numbers of different intracellular signaling pathways regulated by Tyrosine kinases (Trk) receptors. Trk receptors, especially TrkB, are also frequently overexpressed in a variety of human malignant tumors. In this study, we have computationally designed small peptide-based inhibitors of TrkB and investigated their effects on the proliferation and apoptosis of two ovarian cancer cell lines. Molecular docking of TrkB with its ligand and antagonist, BDNF and Cyclotraxin B respectively, was carried out using HADDOCK program. A peptide library was constructed based on the critical residues involved in the TrkB binding site. After docking and optimization, two selected peptides were purchased and their effects on the viability and apoptosis of the cells were evaluated by performing MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) test and flow cytometry assay. Subsequently, the levels of expression and phosphorylation statues of TrkB and its two downstream genes including MAPK3 and eIF4E were assessed with western blot. We found that designed peptides effectively reduced TrkB, MAPK3 and eIF4E phosphorylation, reduced cell viability and induced apoptosis in the treated cells when compared to untreated cells. In conclusion, the BDNF/TrkB signaling is shown to be attenuated substantially in the presence of peptide inhibitors suggesting a strong inhibitory potential of the designed peptides for Trk family.

Keywords TrkB · Cancer · Small peptide · Docking

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Introduction

The Tyrosine kinases (Trk) receptor family is one of the best-known class of transmembrane receptors that is necessary for development and survival of the mammalian nervous system. There are three most common types of Trk receptors including TrkA, TrkB, and TrkC which have different binding affinity to certain types of neurotrophins (Nakagawara 2001). Comparisons of their function indicate that TrkB and TrkC are the receptors for BDNF, NT4 and NT3 respectively, while TrkA is activated by nerve growth factor (NGF). These tyrosine kinase receptors are highly related to different intracellular signaling pathways regulating proliferation, cell survival, axonal and dendritic growth, synapse formation, cytoskeleton remodeling and membrane trafficking (Huang and Reichardt 2003). It has been widely demonstrated that Trk receptors activate both MAPKs and Akt, leading ultimately to further downstream signaling pathways for cell proliferation and development (Jang et al. 2007). However, many studies have reported that skewed population of neurons expresses TrkB, which suggests that this receptor plays a critical role in the regulation of neuronal development in vivo (Li et al. 2012).

Furthermore, other important studies underline the role of TrkB in the invasion ability of cancer cells. Ectopic expression of TrkB and its ligand, BDNF, has been shown to be associated with aggressive tumor behavior and poor prognosis in cancer patients (Okamura et al. 2012). TrkB is also overexpressed by some tumors of the ovarian cancer (Yu et al. 2008), lung cancer (Götz and Sendtner 2014), colorectal cancer (Dawson et al. 2014), neuroblastoma, prostate adenocarcinoma, Wilm's tumors, pancreatic adenocarcinoma and myeloma, and is linked to an increased in anti-cancer drug resistance and tumor invasion (Thiele et al. 2009). Previously, Fujikawa et al. (2012) demonstrated that high TrkB expression is accompanied by epithelial-to-mesenchymal transition (EMT) in colorectal cancer resulting in enhanced tumor metastasis (Fujikawa et al. 2012). Accordingly, TrkB receptor can be considered as a promising target in attempts to inhibit tumor invasion and metastasis. Based on the Trk receptors, some strategies have been proposed for drug discovery to overcome chemotherapy resistance in Trk-associated cancers, but these efforts have not yet been very successful (Maina 2014). Recently, Cazorla et al. designed a potent anti-TrkB inhibitor by using peptidomimetic approach. This small peptide, Cyclotraxin B, could sufficiently alter the conformation of TrkB receptor resulting in the inhibition of BDNF/TrkB signaling and its downstream targets (Cazorla et al. 2010). This study demonstrates that TrkB targeting using peptide-based inhibitors can considerably modulate BDNF/TrkB pathway resulting in a decreased function of proliferation-related genes in TrkB overexpressed cancer cells.

Small peptide-based inhibitors are very interesting candidates for cancer therapy because of their unique physicochemical properties (Wu et al. 2014). TrkB is a well-characterized protein which synthesized as single-pass transmembrane and has been recently considered as a potentially suitable receptor for designing new small peptide antagonists (Cazorla et al. 2010). Although inhibition of TrkB receptor may effectively reduce cancer invasion during cancer progression, most studies have been focused on the TrkB activation in the neural disorders through small-molecule agonists (Jiang et al. 2013; Zeng et al. 2013).

In this study, we designed several BDNF-derived small peptides using molecular docking studies and evaluated their effects on the proliferation and apoptosis of TrkB overexpressed ovarian cancer cells. These peptides were designed based on the binding site of BDNF located at the variable region III of BDNF. Among five top peptides, two peptides were chosen for experimental analysis. These peptides, PEP1 and PEP2, could bind to and significantly inhibit TrkB activity as well as its two downstream genes, MAPK3 and eIF4E, in the ovarian cancer cell lines.

Materials and Methods

In Silico Design of Peptide Inhibitor Using Molecular Docking

A library of the TrkB small peptides was built corresponding to the region III of the BDNF with sequence of N′-Thr^{56}-Lys^{57}-Cys^{58}-Asn^{59}-Pro^{60}-Met^{61}-Gly^{62}-Tyr^{63}-Thr^{64}-Lys^{65}-Glu^{66}-C' using HADDOCK software (De Vries et al. 2007). Preparing of the 3D structures was the starting point for the modeling studies. The crystal structure of both TrkB (PDB code: 1WWB) and BDNF (PDB code: 1B8M) proteins obtained from PDB database (www.rcsb.org). Prior to docking, the 3D structures of proteins were visualized by PyMOL program and unwanted molecules were removed from the PDB files (http://pymol.sourceforge.net). After the study of the interaction of TrkB with BDNF and Cyclotraxin B, the key residues involved in the interaction were determined and provided as input to the Rosetta software for designing new peptides (Rohl et al. 2004). We used the Backrub for flexible peptide-backbone modeling and sequence tolerance protocol for designing the stable peptides. Finally, the models with high scores were selected using the R software (2012). During the backrub protocol many mutations were generated that need to be sorted based on energy score. In order to identify the most stable sequences, we used R software for sorting generated ensembles. The 3D structure of selected peptides was modeled using PEP-FOLD modeling program (Maupetit et al. 2009). Molecular docking studies were carried out using HADDOCK software and schematic representation of docked complexes were generated using the LIGPLOT program (Wallace et al. 1995).The energy value of docked peptides was evaluated and eventually, two peptides with lower energy than others were selected and synthesized by TAG Copenhagen (Copenhagen, Denmark).

Molecular Dynamics Simulation

Molecular dynamics (MD) simulation was carried out using GROMACS (version 4.5) software package (Pronk et al. 2013). The 3D structures of peptide-TrkB complexes were solvated in a solvation box with 10 Å distance between the edges of the box and the protein fragments. In order to neutralize the system, Na^+ and Cl^- ions were added into the system. The equilibrated systems were subjected to 10 ns MD simulation in the isothermal–isobaric (NPT) ensemble using the leap-frog algorithm with an integration time step of 0.002 ps.

Cells and Cell Culture

The SK-OV-3 and OV-CAR-3 cell lines were purchased from American Type Culture Collection (Manassas, VA).

The cells were maintained in RPMI 1640 supplemented with 10 % FBS and 10 μg/ml penicillin. Both cell lines were seeded in their respective growth media and cultured in a humidified atmosphere of 5 % CO_2 and 95 % air at 37 °C.

Cell Growth Assay

The cytotoxic effect of inhibitory peptides on the SK-OV-3 and OV-CAR-3 cancer cell lines was measured using MTT dye reduction assay. The cells (7×10^4 cells/100 μl media) were seeded in 96-well plates and were grown for 24 h. Then, cells were incubated with different concentrations of the inhibitory peptides (50,200,350 and 500 nM) for 24, 48 and 72 h. The plates were then incubated with 100 μl of 0.5 mg/ml MTT solution for 4 h at 37 °C. The formed formazan crystals were dissolved by the addition of 100 μl/well DMSO and the absorbance was measured at 570 nm by a 96-well micro plate ELISA reader. Data were presented as relative percent viability with respect to untreated and Cyclotraxin B-treated cells. The $IC_{50}$was calculated from mean ± SD values.

Apoptosis Analysis

After an overnight incubation, cells were treated with 200 nM control vehicle (Cyclotraxin B) and various concentrations (350 and 450 nM) of inhibitory designed peptides for 24 and 48 h. After treatment, cells were stained with FITC-Annexin V using FITC-Annexin V Detection kit (Biolegend) followed by flow cytometry. Subsequently, samples were washed twice with PBS, resuspended in Annexin-V binding buffer, stained with PI (50 mg/ml) and kept in the dark for 15 min at 20–25 °C. Staining was measured on a FACScan and percentages of apoptotic cells were determined using FACSDiva software (BD Biosciences).

Analysis of TrkB Downstream Signaling Pathways

It has been reported that TrkB is overexpressed more highly in OV-CAR-3 than SK-OV-3 cells (Yu et al. 2008), therefore we decided to evaluate peptides effect on the TrkB signaling pathway in OV-CAR-3 cell line. OV-CAR-3 cells were grown, treated with 350 nM PEP1, PEP2 and Cyclotraxin B for 48 h and lysed in Passive Lysis Buffer. Lysates were centrifuged at $13000 \times g$ for 10 min at 4 °C.Theproteins in the lysate were fractionated on 10 % SDS-PAGE gel, transferred onto PVDF membrane, washed, and blocked in TBS with 3 % BSA. Immunodetection was carried out using specific primary antibodies: TrkB, phospho-TrkB, MAPK3, phospho-MAPK3, eIF4E, phospho-eIF4E (1/1000), and β-actin (1/20,000). The HRP-conjugated secondary antibodies were used at 1:2500 dilutions. All specific primary and secondary antibodies were purchased from GenScript (Piscataway, NJ) and Cell signaling Inc respectively. Untreated cells were taken as negative control and β-actin was used for loading control. The blots were processed with the ECL Plus Western Blotting detection kit (Pierce Biotechnology, Rockford, IL, USA) and the signal detected using an LAS-3000 image analyzer (Fuji Photo Film Co., Tokyo, Japan). Quantification of Western blots was performed using ImageJ software (Rasband 2012). The statistical analyses were performed with SPSS 15.0 software (SPSSInc, Chicago, Illinois) using Student's t tests toanalyze statistical significance.

Statistical Analysis

All experiments were done in triplicate, statistical analysis was performed by using one-way ANOVA and Tukey's post hoc tests ($\alpha = 0.05$).

Result

Peptide Library Construction

A peptide library of random amino acid sequences was constructed based on genetic algorithm. After generating the peptide library using backrub and sequence tolerance protocols, the five most stable peptides were selected based on the energy scores in the R package and subjected to HADDOCK docking program (data not shown). The molecular weight, isoelectric point, instability and other properties of selected peptides were predicted using MolProbility and ProtParam web tools (Table 1).

Study of Molecular Docking of Designed Peptides with TrkB

Initially, 3D structure of selected peptides was predicted using PEP-FOLD modeling software followed by energy minimization (Fig. 1). Automated docking of peptides with TrkB performed using the standard HADDOCK protocol and the energy value of each peptide calculated and listed in Table 2. After docking with TrkB, PEP2 had the lowest energy value among all six analyzed peptides.

The residues of five selected peptides involved in polar and non-polar interactions with TrkB were illustrated using LIGPLOT and tabulated in Table 3. Comparative analysis of HADDOCK result indicated that the designed PEP1 (TGLDSDGLYQN) and PEP2 (NTDLLNSNDNG) had the highest affinity for TrkB among five selected peptides. Therefore, these two peptides were selected for experimental analyses. Molecular docking indicated that PEP1,

Table 1 Predicted properties of five selected peptides using Molprobility and Protparam servers

Peptide name	Peptide sequence	MW	TPI	No. NAA	No. PAA	Chemical formula	GRAVY
PEP1	TGLDSDGLYQN	1182.2	3.56	2	0	$C_{49}H_{75}N_{13}O_{21}$	−0.909
PEP2	NTDLLNSNDNG	1176.1	3.56	2	0	$C_{45}H_{73}N_{15}O_{22}$	−1.391
PEP3	QYQNNQATLDQ	1322.3	3.80	1	0	$C_{54}H_{83}N_{17}O_{22}$	−1.900
PEP4	SSTQDTRQNTS	1224.2	5.55	1	1	$C_{45}H_{77}N_{17}O_{23}$	−2.091
PEP5	DEKFKKQYFYL	1508.7	8.38	2	3	$C_{74}H_{105}N_{15}O_{19}$	−1.400
Cyclotraxin B	CNPMGYTKEGC	1271.4	7.85	1	2	$C_{53}H_{86}N_{14}O_{18}S_2$	−1.373

MW molecular weight, *TPI* theoretical pI, *No. NAA* number of negative amino acids, *No. PAA* number of positive amino acids, *GRAVY* grand average of hydropathicity

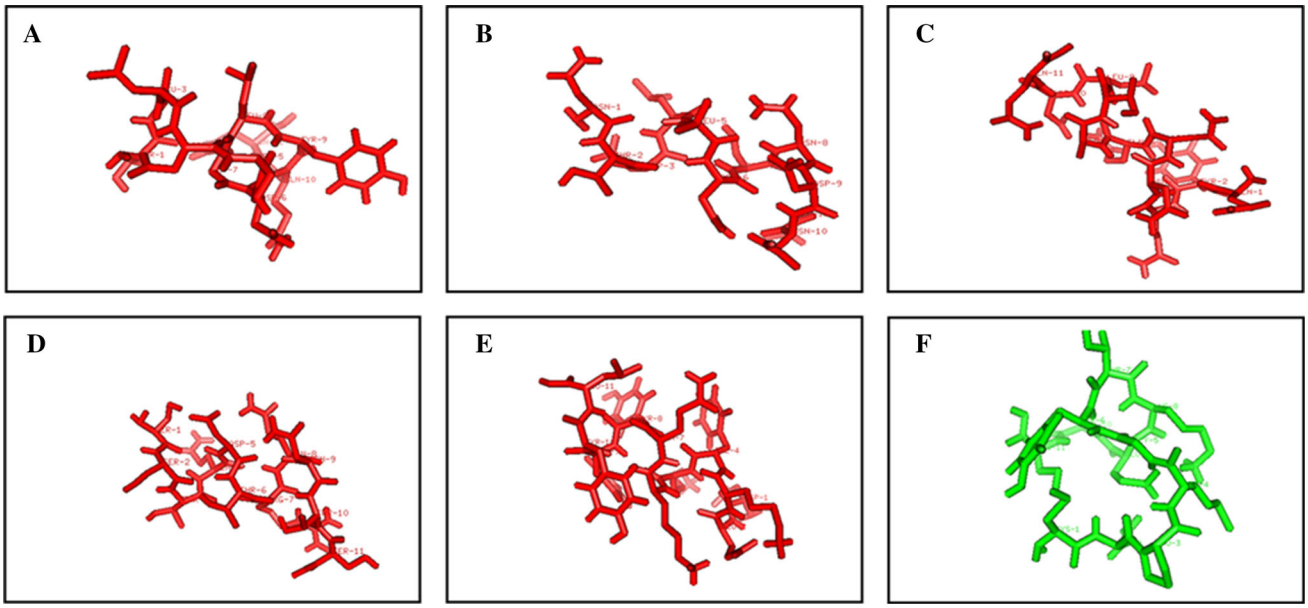

Fig. 1 Predicted structures of five selected peptides and Cyclotraxin B using PEP-FOLD tool. **a** PEP1, **b** PEP2, **c** PEP3, **d** PEP4, **e** PEP5, **f** Cyclotraxin B

Table 2 Docking energy values of selected peptides with TrkB using HADDOCK

Protein	Peptide name	Peptide sequence	Energy (kcal/mol)
TrkB	PEP1	TGLDSDGLYQN	−36.8
	PEP2	NTDLLNSNDNG	−47.9
	PEP3	QYQNNQATLDQ	−20.4
	PEP4	SSTQDTRQNTS	−12.5
	PEP5	DEKFKKQYFYL	−7.1
	Cyclotraxin B	CNPMGYTKEGC	−35.8

PEP2 and Cyclotraxin B had 11, 17 and 14 hydrogen bonds with TrkB respectively. The proposed positioning of PEP1 and PEP2 on TrkB are shown in Fig. 2. Further details of these interactions obtained from LIGPLOT package can be found in the Supplementary Data. The result of molecular dynamics simulation indicated that both PEP1-TrkB and PEP2-TrkB complexes are stabilized after almost 4 ns simulations. The RMSD plot of PEP2-TrkB was even more acceptable than PEP1-TrkB and Cyclotraxin B-TrkB plots (Fig. 3). The two best peptides were purchased from TAG Copenhagen (Copenhagen, Denmark).

Cytotoxic Effect of Designed Peptides on Ovarian Cancer Cell Lines

For assessment of cytotoxic effect of selected peptides, two ovarian cancer cell lines including ov-car-3 and sk-ov-3 were treated with selected inhibitory peptides. The 50 % inhibitory concentration (IC50) of PEP1 and PEP2 were calculated by performing MTT assay (Table 4). The IC50 of PEP2 was lower than Cyclotraxin B indicating higher inhibitory potential of PEP2 against TrkB receptor.

Table 3 Residues of designed peptides interacting with TrkB as predicted by LIGPLOT analysis

Receptor/ inhibitor	Polar interaction forming AAs	Non-bonded contacts forming AAs
TrkB	Lys333, Thr337, Lys312, Lys364, Leu324, Cys331, Lys328, Asp349, Tyr329	His335, Thr332, Ile334, Pro313, Val307, Ala314, Leu315, Gln316, Trp317, Asn325, Glu326, Gln347, Ile330
PEP1	Leu3, Asp4, Asp6, Asn11, Tyr9, Thr1	Gly2, Leu8, Gln10, Gly7
TrkB	Ser297, His299, Asn350, Asp349, Asp298, Gln347, Lys333, Ser327, Thr332, Pro313, Lys312, Leu315	Glu326, Ala314, Val307, Tyr342, Ile334, Cys331, Tyr329, Lys328, Ile330, His300
PEP2	Asn1, Thr2, Asp3, Leu5, Asn6, Asn8, Asn10, Gly11, Asp9	Ser7, Leu4
TrkB	His335, Lys333, Asp349, Lys328, Gln347, Cys331, Glu326, Leu315, Lys312, His339, Gly309	His300, Thr337, Thr340, Tyr342, Val307, Pro313,Ala363, Ala314, Ile334, Thr332, Ser327, Ile330, Tyr329
PEP3	Gln11, Asp10, Thr8, Gln6, Asn5, Gln3, Tyr2, Gln1	Leu9, Asn4, Ala7
TrkB	Ala314, Ile362, Asn325, Ser327, Ile330, Glu326, Lys328, Leu315, Lys312	Pro313, Lys364, Phe305,Tyr34, Gly344, Ile334, Lys333, Thr332, Cys331, Ile323, Gln316, Ala363
PEP4	Leu11, Gln7, Lys5, Asp1, Lys3	Phe4, Glu2, Phe9, Tyr10
TrkB	Asp349, Gln347, His300, His343, Ile334, Cys331, Lys333, Pro313, Glu326, Leu315, Leu324, Ala314, Gln316	Lys364, Trp317, Asn325, Lys312, Lys328, Thr3, Ser327, Gly344, Thr332, Phe305, Leu348
PEP5	Ser2, Ser1, Asp5, Gln4, Thr6, Gln8, Arg7, Ser11	Asn9, Thr10, Thr3
TrkB	Thr332, Ile334, Lys312, Gly344, Val307, Tyr342, Pro313, Leu315, Trp317, Leu324, Asn325, Glu326	Lys333, His343, Phe305, Thr306, Ala314, Gln316, Leu346, Ile323, Cys331
Cyclotraxin B	Asn2, Cys1, Met4, Tyr6, Thr7, Cys11	Glu9, Lys8, Pro3, Gly5, Gly10

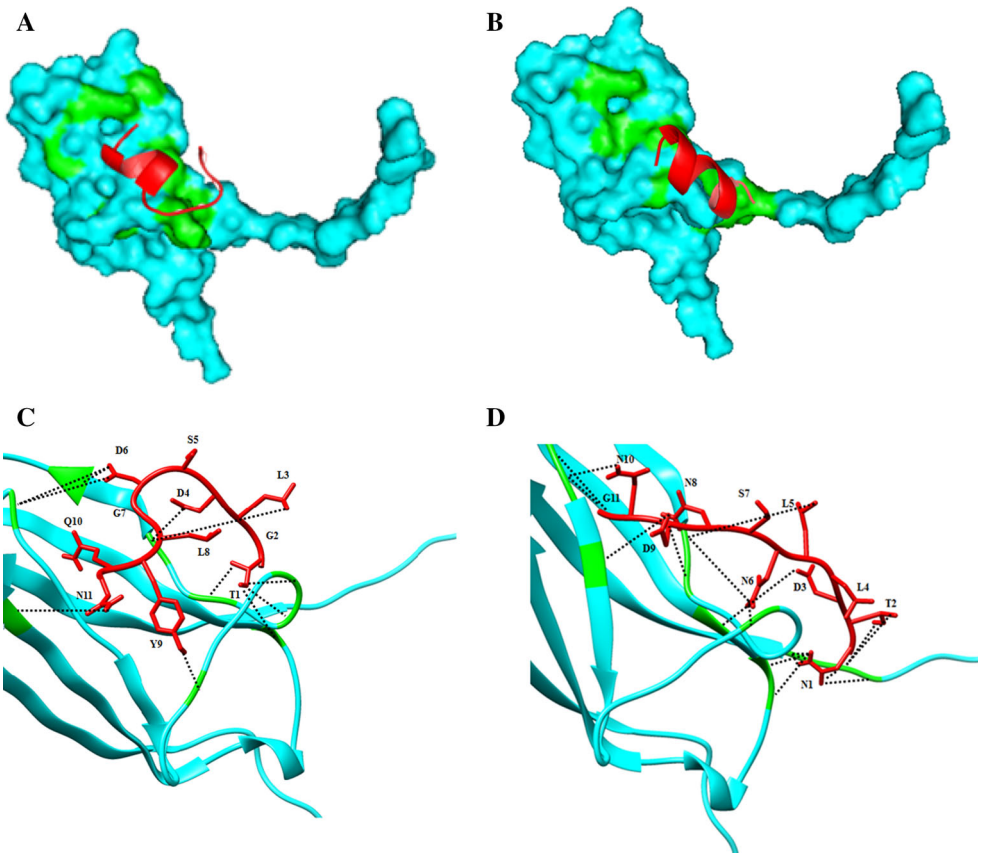

Fig. 2 Positioning of **a** PEP1, **b** PEP2 on the TrkB (*Cyan*) and hydrogen bonds of PEP1 (**c**) and PEP2 (**d**) with TrkB. The peptide inhibitors are shown in the *red color*. Schematic representation of hydrogen bonds between peptides and TrkB are shown in *black dotted lines*. For simplicity, any non-bonded contacts of TrkB with peptides have been omitted. TrkB residues involved in hydrogen bonding with peptides are indicated in *green color* (Color figure online)

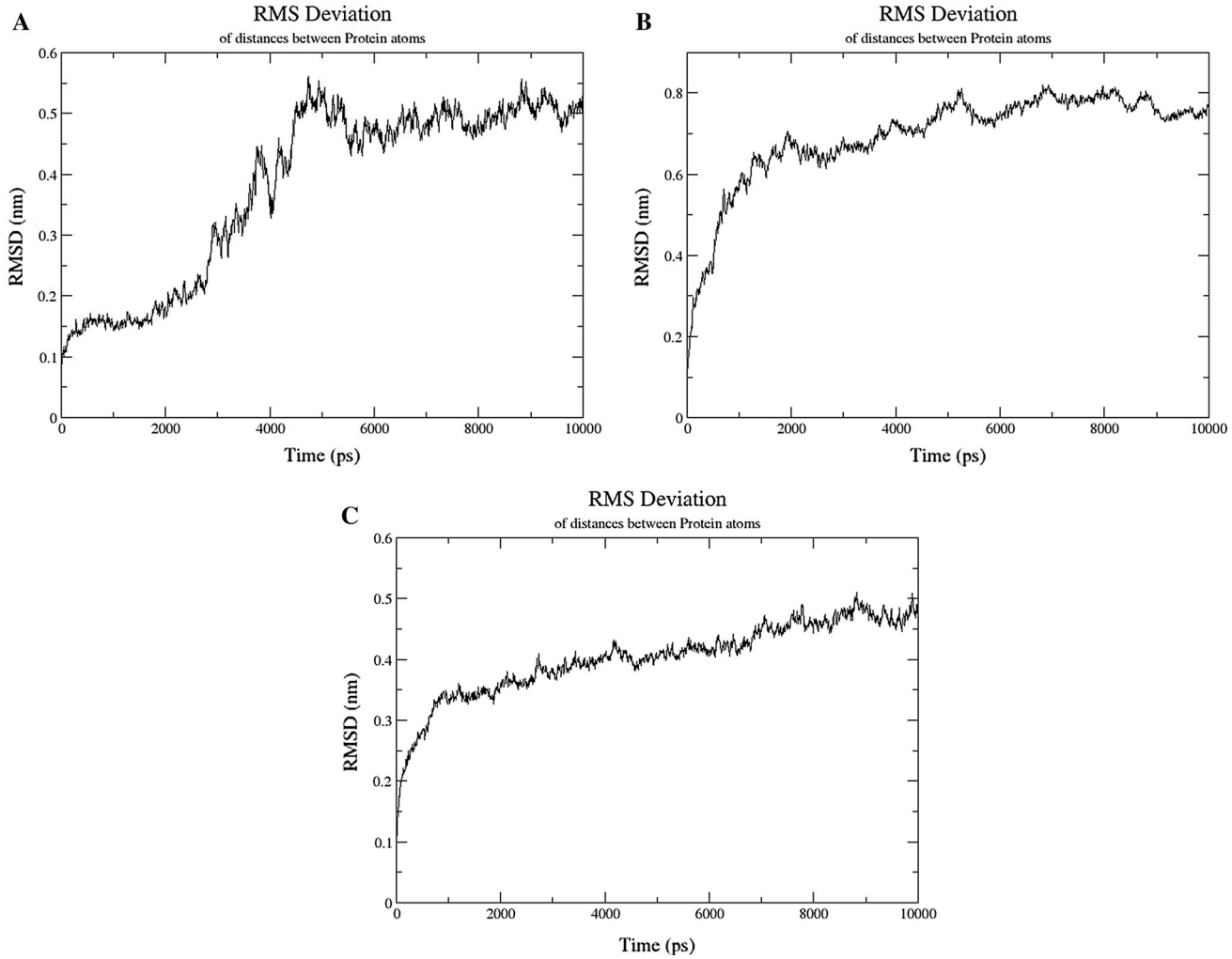

Fig. 3 The RMSD plot of **a** PEP1-TrkB, **b** PEP2-TrkB and **c** Cyclotraxin B-TrkB complexes simulation. The complexes were subjected to a 10 ns MD simulation with an integration time step of 0.002 ps

However, after 24 h treatment of the cells, the IC50 of PEP1 was clearly higher than Cyclotraxin B. Treatment of two ovarian cancer cell lines with inhibitory designed peptides reduced the cell viability significantly (A P value <0.05 was considered significant). Figure 4 summarizes the effect of PEP1 and PEP2 on ov-car-3 and sk-ov-3 cell viability with range 50–500 nM of peptide concentrations after 24, 48 and 72 h treatment.

Table 4 The IC50 of tested inhibitory peptides

Cell line	Peptide name	IC50 (nM)		
		24 h	48 h	72 h
Ov-car-3	PEP1	340.92	199.5	199.21
	PEP2	199.52	198	197.3
	Cyclotraxin B	200	200	200
Sk-ov-3	PEP1	351.02	199.2	199.4
	PEP2	199.45	197.2	198
	Cyclotraxin B	200	200	200

Apoptosis Evaluation in the Presence of Designed Inhibitory Peptides

We evaluated the effect of PEP1 and PEP2 on the apoptosis of ov-car-3 and sk-ov-3 cell lines. After treatment of cells with concentration of 350 and 450 nM designed peptides for 24 and 48 h, we observed slight elevation in the amount of apoptotic cells. Approximately in both 24 and 48 h after the treatment with concentration of 350 and 450 nM peptides, the apoptotic cells were obviously increased. Further details are shown in Table 5. The highest effect of peptides on the induction of apoptosis were observed in the ov-car-3 cell line compared with sk-ov-3 cell line, especially after 24 h treatment with PEP1 (31.59 and 32.21 % apoptotic cells at concentration of 350 and 450 nM respectively). These results confirm that the designed inhibitory PEP1

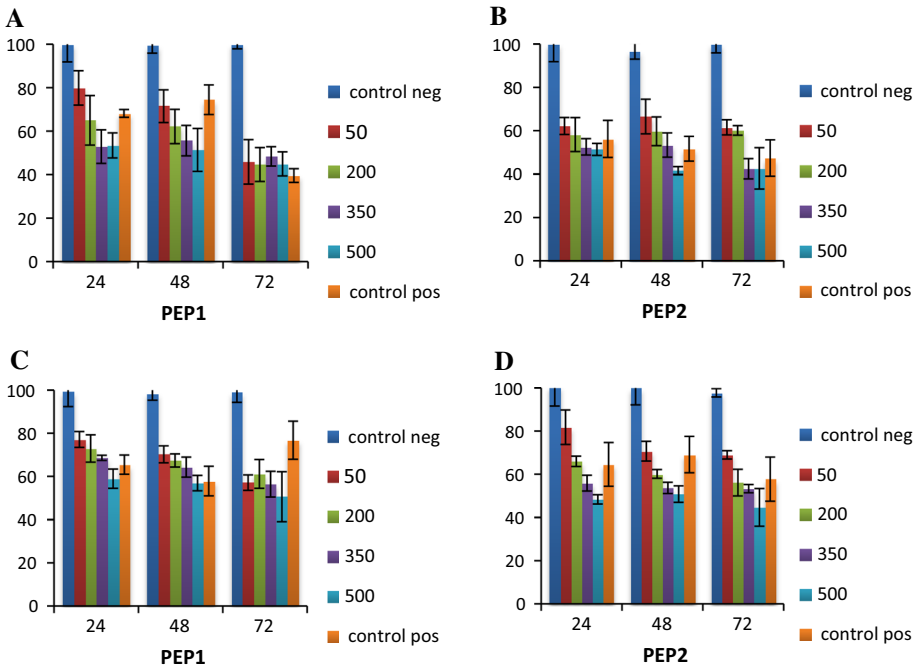

Fig. 4 The effects of PEP1 and PEP2 on the ov-car-3 and sk-ov-3 cell viability. The viability of two ovarian cancer cell lines reduced at different concentrations (50–500 nM) of designed peptides after 24, 48 and 72 h treatment. **a** and **b** ov-car-3 cell line, **c** and **d** sk-ov-3 cell line. Control neg: untreated cells, Control pos: Cyclotraxin B-treated cells (Color figure online)

Table 5 Apoptosis percentage of treated cells with different concentrations of PEP1, PEP2 and Cyclotraxin B

Cell line	Peptide name	Peptide concentration (nM)	Apoptosis percentage (%)	
			24 h	48 h
Ov-car-3	PEP1	350	31.59	17.9
		450	32.21	24.99
	PEP2	350	23.18	20.79
		450	25.33	28.39
	Cyclotraxin B	200	19.41	20.85
	Negative control		2.82	2.82
Sk-ov-3	PEP1	350	18.07	20.11
		450	22.18	25.14
	PEP2	350	14.67	23.98
		450	24.22	23.53
	Cyclotraxin B	200	20.56	16.47
	Negative control		2.32	2.32

and PEP2 are capable of inducing apoptosis in the ovarian cancer cells.

Analysis of TrkB Downstream Signaling Pathways

The main importance of BDNF/TrkB signaling is that this signaling can regulate the function of some proliferation-related proteins such as MAPKs and eIF4E through directly phosphorylation of these proteins. Western blotting result demonstrated that phosphorylation of TrkB, MAPK3 and eIF4E proteins declined in the treated cells when compared to control cells (Fig. 5). However, the total level of these proteins in the treated cells did not reduce significantly. The inhibition effect of PEP1 and PEP2 on the phosphorylation of aforementioned proteins, especially on the TrkB, were considerably higher than Cyclotraxin B. All three peptides (PEP1, PEP2 and Cyclotraxin B) reduced TrkB phosphorylation significantly. In addition, PEP2 also caused a significant reduction in eIF4E phosphorylation.

Discussion

In contrast to larger molecules, small peptide-based drugs would represent more appropriate treatment in cancer therapy because of their smaller size, excellent tissue

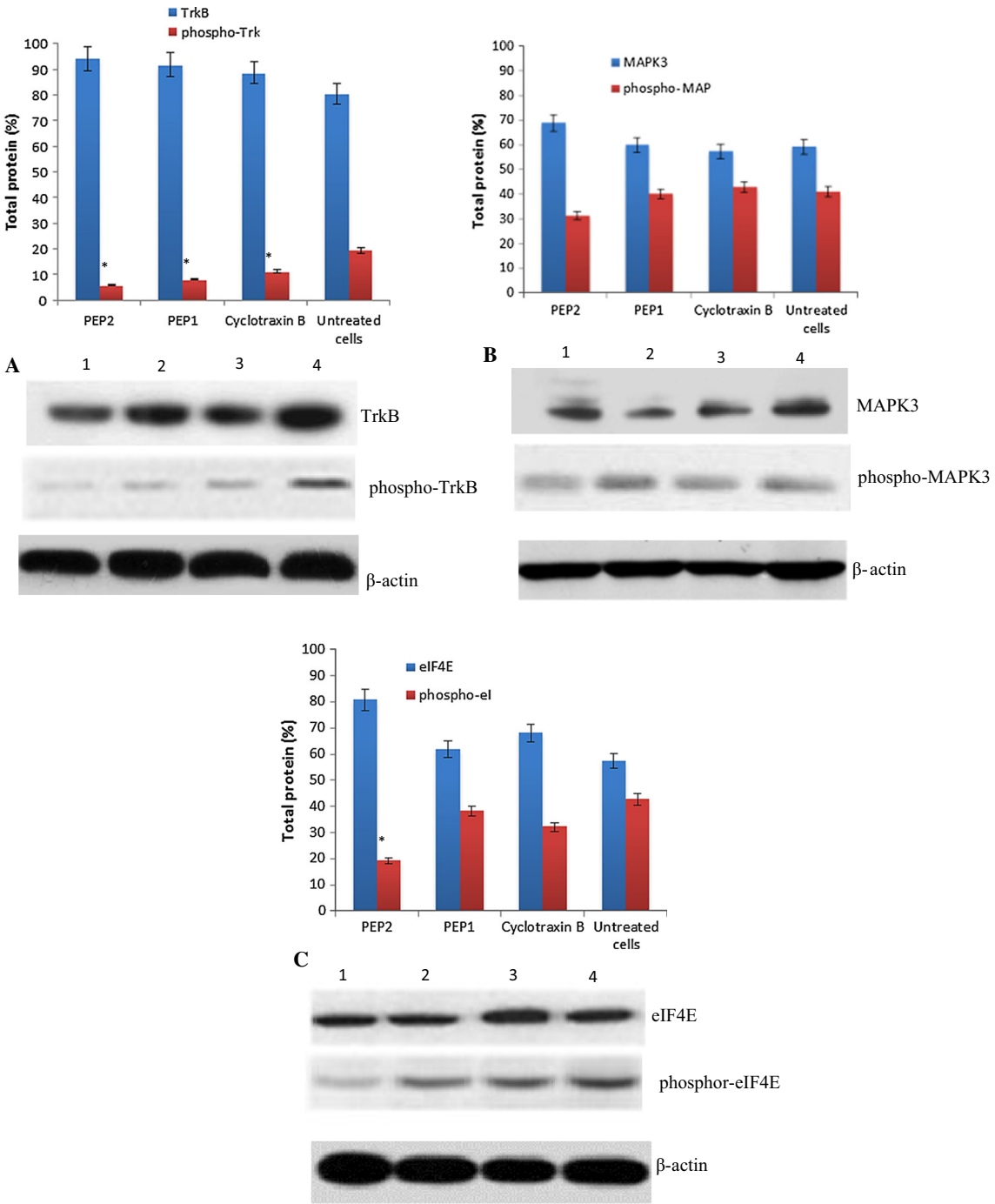

Fig. 5 Western blot analysis of total and phosphorylated level of TrkB and its downstream genes. **a** (*Up* anti-TrkB antibody, *down* anti-phosphoTrkB antibody), **b** (*up* anti-MAPK3 antibody, *down* anti-phosphoMAPK3 antibody), **c** (*up* anti-eIF4E antibody, *down* anti-phosphoeIF4E antibody). *Lane 1* PEP2, *lane 2* PEP1, *lane 3* Cyclotraxin B, *lane 4* untreated cells. β-actin expression levels in the sample were used as the loading control. Blots were quantified and shown above each *panel*. PEP2 reduced phosphorylation of TrkB and eIF4E significantly (*P* value <0.05) (Color figure online)

penetration and easy synthesis process. There are several small peptides, also known as cell-penetrating peptides, that unlike other biotherapeutic agents can efficiently translocated through the plasma membrane. These peptides are widely considered as one of the most promising tools for delivering numerous types of drugs (such as small-molecule pharmaceuticals, therapeutic proteins, and anti-sense oligonucleotides) into target cells (Stewart et al. 2008). One of the most important challenges in peptide-based drug design is to find a promising therapeutic target

associated with cancer progression. It has been shown that BDNF/TrkB signaling is involved in the pathogenesis of different cancers mainly in ovarian cancer (Au et al. 2009) multiple myeloma (Hu et al. 2007) and colon cancer (Yu et al. 2010). Also, overexpression of TrkB has been reported in several human malignant tumors such as neuroblastoma which is likely to play an unfavorable role in neuroblastoma resistance to chemotherapy-induced apoptosis (Jaboin et al. 2002). There are some of the TrkB inhibitors which effectively inhibit BDNF/TrkB signaling in cancer cells and subsequently prevent cancer cell invasion (Desmet and Peeper 2006). AZ623, a TrkB inhibitor, in combination with another chemotherapy drug (topotecan) at concentration of 0.8 to 7 μM could inhibit TrkB-mediated signaling in neuroblastoma (Zage et al. 2011). Cazorla et al. indicated that the concentration of 200 nM Cyclotraxin B efficiently inhibited TrkB receptor in vivo (Cazorla et al. 2010). In this study, we considered Cyclotraxin B as positive control. We introduced new potentially small peptide-based inhibitors of TrkB which could strongly modulate BDNF/TrkB signaling, even at lower concentrations compared to Cyclotraxin B. The results of molecular docking studies revealed that PEP2 had a moderately strong Hydrogen-Bond pattern which results in a tight interaction with TrkB. The Asn1 residue of PEP2, by making a 6 hydrogen-bonds pattern with Ser297, Asp298, His299, Asp349 and Asn350 residues of TrkB, plays a very important role in the interaction of PEP2 with TrkB (Fig. 6). Generally, asparagine has potential hydrogen bonding atoms and plays a critical role in many enzymes functions and also helps protein refolding by interacting, directly or indirectly, with the hydrophilic groups (Choma et al. 2000). There are four asparagine residues in the sequence of PEP2 which may make a better binding affinity of this peptide to TrkB receptor.

The results of molecular docking revealed that the Lys333 residue of TrkB plays a key role in the binding of peptides to the TrkB. The Lys333 residue, in Cyclotraxin B-TrkB complex, makes a non-bonded contact with TrkB, while this residue makes two hydrogen bonds with both PEP1 (with Leu3 and Asp4 residues) and PEP2 (with Leu5 and Asn6 residues). It is now well established that a combination of Leu with either Asn or Asp residues is highly effective in inhibiting protein degradation (CARO et al. 1989). Combination of Leu with Asp and Asn in PEP1 and PEP2 respectively, may enhance the therapeutic effectiveness of these peptides by improving their stability.

Characterization of ligand-binding domain of the Trk family members have demonstrated that the hydrophilic residues in the Trk family members play a key role in the binding of ligands to Trk kinases (Ultsch et al. 1999). Similarly, the main candidate region of BDNF involved in the interaction with its receptor is surrounded by charged and

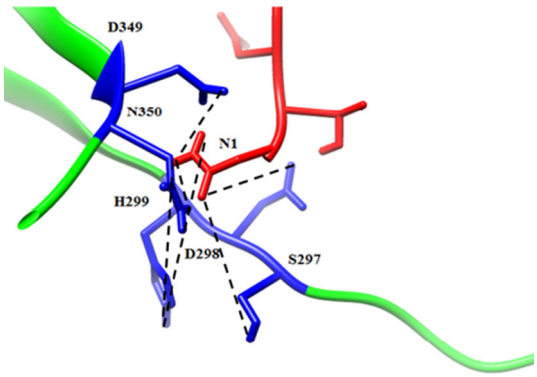

Fig. 6 The six hydrogen-bonds pattern of Asn1 residue of PEP2 (*red color*) interacted with TrkB. The residues of TrkB involved in the interaction with Asn1 residue of PEP2 are shown in *blue color* (Color figure online)

specificity-conferring residues (Robinson et al. 1999). These studies indicate that the binding of BDNF to TrkB is highly dependent on polar interactions between hydrophilic residues. The physicochemical properties of selected peptides and Cyclotraxin B calculated by Peptide property calculation tool of Innovagen (http://www.innovagen.se) showed a remarkable hydrophilicity scale of PEP2 which might explain better binding capacity of PEP2 than PEP1 and Cyclotraxin B (data not shown). Furthermore, the IC50 values of PEP2 were lower in 24, 48 and 72 h than Cyclotraxin B in the two treated ovarian cancer cell lines. The lower IC50 values indicate the greater potency of PEP2 in antagonizing the BDNF/TrkB signaling. Analysis by Western blotting indicated that PEP2 could significantly inhibit the phosphorylation of TrkB and its downstream genes.

TrkB triggers some downstream signaling pathways associated with cell growth and development. Activation of MAPKs cascade can be achieved by Trk receptor activation and the vast majority of evidences have demonstrated that MAPKs activation could increase cell migration in non-invasive cancer cells (Krueger et al. 2001). There are several new strategies for design of improved therapeutic approaches based on MAPKs function model in the cell (De Luca et al. 2012). Recent studies have reported that mTOR signaling pathway acts as a node of convergence down-stream of BDNF/TrkB (Slipczuk et al. 2009). BDNF/TrkB-induced increase of mTOR stimulates synthesis of proteins through regulation of p70S6K and 4E-BP1 (Park et al. 2014). Indeed, activated mTOR phosphorylates and inactivates 4E-BP1 by repressing its binding to eIF4E (Troca-Marín et al. 2012). Then, free eIF4E could bind to the eIF4G and eIF4A and form eIF4F active complex facilitating translation initiation. In this study, we found that the phosphorylation of MAPK3 and eIF4E declined after treatment of the cells with TrkB designed inhibitors. Among three evaluated peptides, PEP2 could significantly inhibit the phosphorylation of eIF4E but there was no significant reduction in the phosphorylation of

MAPK3 after treatment with inhibitory peptides. On the basis of recent studies, it is reasonable that the inhibition of TrkB receptor would not be expected to significantly modulate the function of MAPK signaling pathway. MAPKs are involved in many biological processes in a wide range of eukaryotic organisms. MAPK signaling pathway, which functions as one of the cell hub signaling pathways, is regulated by many other signaling pathways such as Tumor necrosis factor (TNF) (Chen et al. 2007), hypoxia (Risbud et al. 2005) and RAS/RAF (Hatzivassiliou et al. 2010). There is a large-scale study that introduces a list of 57 different genes activating MAPK signaling pathway (Matsuda et al. 2003). However, our results are clearly compatible with previous studies that have found the reduction in phosphorylation of TrkB accompanied by reduced downstream genes phosphorylation (Cazorla et al. 2010).

Up-regulation of TrkB can promote cell proliferation (Heo et al. 2013) and chemotherapy resistance (Lee et al. 2012) through inhibiting apoptosis. Expectedly, inhibition of TrkB by designed peptides slightly increased the amount of apoptosis in the set of cell lines studied here. Since inhibition of TrkB cannot completely block the anti-apoptotic function of BDNF (Takeda et al. 2013), we observed only a slight increase in the apoptotic cell population.

In conclusion, the aim of present study was to demonstrate that inhibition of TrkB by peptide-based inhibitors can considerably down-regulate its downstream target genes including MAPK3 and eIF4E. In fact, the modulating of these genes may enhance the chemotherapy responses in cancer cells through regulation of cell death. Therefore, the TrKB inhibition by means of rational designed peptides would be useful in control of that pathway.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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